CHAPTER 3

The effect of different hormonal contraceptives on plasma levels of free Protein S and free TFPI

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Abstract

Background:

Use of combined oral contraceptives is associated with a three- to six-fold increased risk of venous thrombosis. Hormonal contraceptives induce acquired resistance to activated protein C (APC), which predicts the risk of venous thrombosis. The biological basis of the acquired APC resistance is unknown. Free protein S (PS) and free Tissue Factor Pathway Inhibitor (TFPI) are the two main determinants APC. Our objective was to assess the effect of both hormonal and non-hormonal contraceptives with different routes of administration on free TFPI and free PS levels.

Methods:

We conducted an observational study in 243 users of different contraceptives and measured APC sensitivity ratios (nAPCsr), free TFPI and free PS levels.

Results:

Users of contraceptives with the highest risk of venous thrombosis as reported in recent literature, had the lowest free TFPI and free PS levels, and vice versa, women who used contraceptives with the lowest risk of venous thrombosis had the highest free TFPI and free PS levels. An association was observed between levels of free TFPI and nAPCsr, and between free PS and nAPCsr.

Conclusion:

The effect of oral contraceptives on TFPI and PS is a possible explanation for the increased risk of venous thrombosis associated with oral contraceptives.

Introduction

Use of combined oral contraceptives is associated with a three- to six-fold increased risk of venous thrombosis. This increased risk depends on the estrogen dose as well as the progestogen type of oral contraceptives (1). So called 'high-dose' combined oral contraceptives containing 50 µg or more ethinylestradiol are associated with a two-fold higher risk of thrombosis than 'low-dose' combined oral contraceptives containing 20 to 30 µg ethinylestradiol (2;3). Further, third-generation combined oral contraceptives, containing the progestogens gestodene or desogestrel, and combined oral contraceptives containing cyproterone acetate or drospirenone increase the risk of venous thrombosis by a factor of two compared with combined oral contraceptives containing levonorgestrel (1;3;4).

Use of combined oral contraceptives causes changes in procoagulant, anticoagulant and fibrinolytic parameters (5). The thrombin generation-based APC resistance test is a global assay that is sensitive to the levels of individual clotting factors and combines these into a net effect (6-8). In line with epidemiological observations, users of 'high risk' combined oral contraceptives containing desogestrel, cyproterone actetate and drospirenone have been found more resistant to the anticoagulant action of APC in this test than users of 'low risk' combined oral contraceptives containing levonorgestrel. (3;4;6;8-11).

The two main determinants of the thrombin generation-based APC resistance test are free protein S (PS) and free tissue factor (TF) pathway inhibitor (TFPI) (12-14). The differences in APC-resistance induced by oral contraceptives can at least in part be explained by different effects on free PS and free TFPI levels (12).

TFPI is a Kunitz-type protease inhibitor that down-regulates the extrinsic (TF-induced) coagulation pathway. It is mainly synthesized by endothelium cells (15). Approximately 80% of intravascular TFPI is bound to the vessel wall, whereas 20% circulates in plasma. Only 10% (~0.25 nM) of TFPI in plasma circulates as free full length TFPI, which is the most active form. TFPI down-regulates thrombin generation by inhibiting activated factor X (FXa) via the formation of a TFPI/FXa complex which subsequently inhibits the TF/activated factor VII (TF/FVIIa) complex by forming an inactive TFPI/FXa/TF/FVIIa quaternary complex (16). TFPI is a slow inhibitor of thrombin generation, which is most effective at low TF concentrations or when thrombin generation is slowed down through the intrinsic pathway (17).

PS is a vitamin K dependent protein which acts as a non-enzymatic cofactor of APC in the proteolytic inactivation of the activated factors Va (FVa) and VIIIa (FVIIIa) (18). It is synthesized in the liver and in endothelial cells. Approximately 60% of the PS in plasma is bound to C4b Binding Protein (C4BP) in a high affinity complex, while the remaining 40% circulates in plasma in a free form. It was recently discovered that, besides its co-factor function with APC, free PS forms a complex with TFPI and stimulates formation of the TFPI/FXa complex, thus enhancing the down-regulation of thrombin generation by TFPI (17;19-23).

Both hereditary and acquired PS deficiency is associated with an increased risk of venous thrombosis (20;21;21). It was observed by Castoldi *et al.* that PS deficient individuals also have decreased TFPI levels, probably due to common mechanisms regulating biosynthesis of both proteins (20). Van Vliet *et al.* (12) observed that women using combined oral contraceptives with the highest risk of venous thrombosis (e.g. containing desogestrel, cyproterone actetate or drospirenone), have lower levels of protein S and TFPI than women using the combined oral contraceptive with the lowest risk of venous thrombosis (i.e. contraceptives containing levonorgestrel).

The aim of this study was to assess if the different risks of venous thrombosis caused by different hormonal and non-hormonal methods of contraception with various routes of administration are reflected in the levels of APC resistance, and free TFPI and free PS, the main determinants of the thrombin generation-based APC resistance test.

Material and Methods

Study design and participants

We conducted an observational study. In a series of four different studies we collected samples of users of different hormonal and non-hormonal contraceptives, including oral, transdermal and vaginal combined hormonal contraceptives, the levonorgestrel-releasing intrauterine device (IUD), the copper-releasing IUD and female non-users with regular, ovulatory menstrual cycles (24-27).

Inclusion criteria of all participants were: healthy women using a hormonal contraceptive for at least three cycles. Exclusion criteria were age <18 years, and contraindications for combined hormonal contraceptive use as stated by the World Health Organization (28). A more detailed description of the studies can be found in the original articles (24;26;27;29).

In total, we excluded 73 participants out of 316 eligible participants. Participants who were carriers of the factor V Leiden or the prothrombin 20210A mutation were excluded from the analysis, because these mutations affect resistance to APC (n = 31). Users of oral contraceptives containing 20 µg ethinylestradiol and groups with a small sample size were excluded: users of the vaginal ring containing ethinylestradiol and etonogestrel (n = 4), users of the transdermal patch containing ethinylestradiol and norelgestromine (n = 3), users of a combined oral contraceptives containing 100 µg levonorgestrel and 20 µg ethinylestradiol (n = 4), 150 µg desogestrel and 20 µg ethinylestradiol (n = 12), 75 µg gestodene and 20 µg ethinylestradiol (n = 7), 75 µg gestodene and 30 µg ethinylestradiol (n = 3), 250 µg norgestimate and 35 µg ethinylestradiol (n = 1) or containing 1 mg norethisterone and 35 µg ethinylestradiol (n = 2). Six participants were excluded because their data were not complete.

In our final analysis, we used the samples of 243 participants: 153 users of a combined oral contraceptive containing ethinylestradiol and levonorgestrel, desogestrel, cyproterone actetate or drospirenone, 60 users of the levonorgestrel-IUD, 17 users of the copper-IUD and 13 non-users (mid-cycle).

Written informed consent was given by all participants and the studies were all approved by the Medical Ethics Committee of the Leiden University Medical Center, The Netherlands.

Laboratory methods

The samples from the studies were drawn, processed and stored identically. Blood samples were taken from the antecubital vein in the morning in a fasting state and collected in 0.106 M sodium citrate (pH 5.8). Cell-free, citrated plasma was prepared by centrifuging blood at 2,100 g for 10 minutes at 18ºC, coded and centrally stored at -80 ºC.

Protein S

Free Protein S was determined by ELISA according to Giri *et al*. (30) with modifications as follows: microtiter plates were coated overnight at 4-8 ºC with purified C4BPß (3,5 µg/mL in 0.1 M NaHCO3, 0.5 M NaCl buffer, pH 9). Wells were washed four times with buffer A (0.05 M Tris-HCl, 0.1 M NaCl, 0.1% Tween, 0.05% ovalbumin, pH 7,5) and incubated with buffer A containing 2,5% ovalbumin for 1 hour at 37 ºC to reduce background absorbance. After four washes with buffer B (0.05 M Tris-HCl, 0.1 M NaCl, 0,1% Tween, 0.05% ovalbumin, 0.005 M CaCl2, 0.01 M benzamidine-HCl, pH 7,5) the dilutions of calibrator (1/10-1/640) and test samples (1/20 and 1/40) were added and incubated for 15 minutes at room temperature. After four washes with buffer B, HRP-conjugated anti-human protein S (0.4 µg/mL) antibody was added and incubated for 1 hour at 37 ºC followed by four washes with buffer B. Subsequently, TMB (0.2 mg/mL) and H2O2 (0.01%) were added and incubated for 15 minutes at room temperature, after which the reaction was stopped by adding 4N H2SO4 and the absorbance at 450 nm was measured. Supernatant of PNP supplemented with an equal volume of 10% PEG 6000 was used as calibrator. This plasma contained 0.28 U/mL of protein S total, which is all free protein S. All sample dilutions were prepared within 10 minutes of starting the assay. Pooled normal plasma contained 0.31 U/mL free protein S and 1.0 U/mL total protein S. Inter- and intra-assay variability was 5.7% and 8.0% respectively.

TFPI

Free TFPI and total TFPI were assayed with commercial enzyme-linked immunosorbent assays (Asserachrom® free TFPI and Asserachrom® Total TFPI, Diagnostica Stage, Asnière, France) as described in detail elsewhere (31). Freeze-dried human plasmas containing known amounts of TFPI provided in the kits were used for calibration. Quality controls were performed using a control specimen containing a high amount of TFPI as well as a sample with normal level of TFPI. Inter- and intra-assay variability, measured as coefficients of variation, was 6,0% and 2,6% for total TFPI, and 9,5% and 5,3% for free TFPI, respectively.

APC resistance

Normalized APC sensitivity ratios (nAPCsr) were determined in duplicate by quantifying the effect of APC on thrombin generation in the thrombin generation-based APC resistance test, as described before (32).

Statistical analysis

In this observational study, we used means, mean differences, 95% confidence intervals of the mean and ranges to describe variables. Scatter diagrams and regression lines were constructed and a regression coefficient with 95% CI was estimated with free PS or free TFPI as independent variable and nAPCsr as dependent variable. A bar diagram was constructed to visualize the association between the risk of venous thrombosis during use of hormonal contraceptives as described in recent literature and the measured free TFPI and free PS levels. Statistics were computed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

There were no differences in BMI and age between the women using different kinds of hormonal contraceptives (Table 1).

Table 1: *Characteristics of participants after exclusion*

Contraceptive	Progestogen	Estrogen	n	Age (years)		BMI (kg/m2)	
				Mean	Range	Mean	Range
None	-	-	13	29	20-48	22	19-26
Cu-IUD	-	-	17	33	20-45	24	18-32
LNG-IUD	20 µg/day LNG	-	60	33	17-52	25	18-48
Oral contraceptive	150 µg/day LNG	30 µg/day EE	70	29	18-51	23	17-38
Oral contraceptive	150 µg/day DSG	30 µg/day EE	16	31	18-49	25	20-32
Oral contraceptive	3 mg/day DRSP	30 µg/day EE	46	29	18-47	24	18-34
Oral contraceptive	2 mg/day CPA	35 µg/day EE	21	28	19-44	22	19-26
Total			243	30	17-52	24	17-48

Free TFPI levels during use of hormonal contraceptives compared with non-use

Women using the levonorgestrel-IUD had higher free TFPI levels than non-users (5.48 ng/ml versus 4.62 ng/ml; difference 0.85 ng/ml, 95% CI 0.05 to 1.66). The mean free TFPI level of women using the copper-IUD did not differ with the mean free TFPI levels of non-users (5.06 ng/ml versus 4.62 ng/ml; difference 0.44 ng/ml, 95% CI -0.52 to 1.39).

Combined oral contraceptive users had lower free TFPI levels than non-users. Women using cyproterone actetate / ethinylestradiol had the lowest mean free TFPI level: 2.51 ng/ml, difference -2.12 ng/ml compared with non-users (95% CI -2.73 to -1.50). (Table 2)

Free PS levels during use of hormonal contraceptives compared with non-use

Women using the levonorgestrel-IUD had similar free PS levels as non-users (0.28 U/dl versus

0.28 U/dl; MD -0.001 U/dl, 95% CI -0.03 to 0.03). The mean free PS levels of women using the copper-IUD did also not differ from non-users (0.26 U/dl versus 0.28 U/dl; MD -0.02 U/dl 95% CI -0.07 to 0.02).

Women using combined oral contraceptives had lower free PS levels than non-users. The lowest mean free PS levels were measured in women using cyproterone actetate / ethinylestradiol: 0.19 U/dl , MD -0.09 U/dl compared with non-users (95% CI -0.12 to -0.05). (Table 3)

Free TFPI and free PS levels compared with levonorgestrel / ethinylestradiol

Women using combined oral contraceptives with a high risk of venous thrombosis had lower free TFPI levels than women using combined oral contraceptives with a lower risk of venous thrombosis, e.g levonorgestrel / ethinylestradiol. The mean difference between levonorgestrel / ethinylestradiol compared with other oral contraceptive users was -1.01 ng/ml (95% CI -1.81 to -0.22) for desogestrel / ethinylestradiol, -0.57 ng/ml (95% CI -1.10 to -0.03) for drospirenone / ethinylestradiol and -1.42 ng/ml (95% CI -2.12 to -0.72) for cyproterone actetate / ethinylestradiol.

The same pattern was observed for free PS levels: the mean difference in free PS of levonorgestrel / ethinylestradiol compared with desogestrel / ethinylestradiol was -0.09 U/dl (-0.12 to -0.05), compared with drospirenone / ethinylestradiol was -0.08 U/dl (95% CI -0.11 to -0.06) and compared with cyproterone actetate / ethinylestradiol was -0.13 U/dl (-0.16 to -0.10).

Association between free TFPI and APC resistance and free PS and APC resistance

In previous reports (33;34) on the resistance to APC in the same study population, we observed higher nAPCsr in the groups of women using combined oral contraceptives containing desogestrel, drospirenone and cyproterone actetate than in non-users. The nAPCsr in women using the levonorgestrel-IUD was decreased compared with non-users and users of the combined oral contraceptive containing levonorgestrel / ethinylestradiol (24).

Free TFPI levels were negatively correlated with the nAPCsr: a linear association was observed with the equation free TFPI = 6.082 – 0.629 * nAPCsr. Thus, a decrease of free TFPI with 1 ng/ml was associated with an increase of nAPCsr with 0.629 (95% CI 0.47 to 0.79).

Free PS levels were also negatively associated with the nAPCsr: a linear association was found with the equation free PS = 0.355 – 0.028 * nAPCsr: when free PS levels decreased with 1 U/dl nAPCsr increased with 0.028 (95% CI 0.022 to 0.34).

Risk ranking per contraceptive

For risk ranking, we used recent publications from van Hylckama Vlieg *et al*. (3;35) (Table 4). The odds ratio for venous thrombosis during use of the levonorgestrel-IUD compared with non-users was 0.3 (95% CI 0.1 to 1.1) (35). The risk of venous thrombosis during use of a copper-IUD is unknown, but expected not to be increased compared with non-users.

Free TFPI levels measured in this study are associated with the odds ratios reported in recent literature i.e lower free TFPI levels were present in users of contraceptives with a higher risk of venous thrombosis. Free PS showed a less pronounced, but similar pattern (Figures 1 and 2).

Total TFPI

Total TFPI levels in plasma of users of different kinds of contraceptives were comparable. No association was observed between known risk ratios and total TFPI levels (data not shown).

Discussion

In this study on the effect of different contraceptives on the two main determinants of the thrombin generation-based APC resistance test, we observed that different doses of ethinylestradiol and different types of progestogens have different effects on the plasma levels of free TFPI and free PS. Women who are according to recent literature exposed to the highest risk of venous thrombosis during use of hormonal contraceptives (i.e. users of combined oral contraceptives containing drospirenone / ethinylestradiol, cyproterone actetate / ethinylestradiol or desogestrel / ethinylestradiol), had the lowest free TFPI and free PS levels and vice versa: women who used hormonal contraceptives with the lowest risk of venous thrombosis (i.e. users of the levonorgestrel-IUD) had the highest free TFPI and free PS levels. An association was observed between free TFPI and nAPCsr, and between free PS and nAPCsr.

The observed effect of different types of oral contraceptives on free TFPI are consistent with a study by Alhenc-Gelas *et al*. (37). Plasma levels of free TFPI were found to be more decreased in women using oral contraceptives containing desogestrel / ethinylestradiol or cyproterone actetate / ethinylestradiol than in women using oral contraceptives containing levonorgestrel / ethinylestradiol.

Several studies observed that third generation oral contraceptives containing gestodene or desogestrel combined with ethinylestradiol induce a larger decrease of free PS than oral contraceptives containing levonorgestrel / ethinylestradiol (38-40). In our study similar decreases in free PS levels were found for desogestrel / ethinylestradiol users compared with levonorgestrel / ethinylestradiol users. In a trial by Kluft *et al*. (41), oral contraceptives containing drospirenone / ethinylestradiol and oral contraceptives containing desogestrel / ethinylestradiol led to similar reductions in PS total antigen levels and PS activity levels, but no data are available on free PS levels. No studies have been published on the effect of cyproterone actetate / ethinylestradiol on free PS levels .

In our study, sample sizes of the groups of users of the vaginal ring containing ethinylestradiol and etonogestrel and users of the transdermal patch containing ethinylestradiol and norelgestromine were too small for analysis. In a randomized controlled trial by Johnson *et al*. (42) patch users had lower free PS levels than users of a levonorgestrel / ethinylestradiol containing oral contraceptive. Jensen *et al*. (43) found that free PS levels of ring users were higher, and free PS levels of patch users were lower compared with use of a combined oral contraceptive. Recently, Lidegaard *et al*. (36) reported a RR of 7.9 (95% CI 3.5 to 17.6) for users of the transdermal patch and a RR of

6.5 (95% CI 4.7 to 8.9) for users of the vaginal ring compared with non-users. Both contraceptives contain an estrogen and a progestogen compound and work systemically. Increased relative risks were observed, so decreased levels of TFPI and PS are to be expected.

The use of the levonorgestrel-IUD is not associated with an increased risk of venous thrombosis (35;44). In this study, women using the levonorgestrel-IUD had higher free TFPI and similar free PS and nAPCsr levels as non-users, indicating that use of the levonorgestrel-IUD is not associated with an increased risk of venous thrombosis. It can be hypothesized that the differences in free TFPI levels are attributable to the progestogen compound of the hormonal contraceptive, since the amount of ethinylestradiol of hormonal contraceptives used in this study were similar while the progestogen types differed, and different levels of free TFPI were observed. Possibly, progestogens cause an increase in free TFPI, as observed in users of the levonorgestrel-IUD, and ethinylestradiol causes a decrease in free TFPI, reflected as a net effect. No other studies have been published on free PS and free TFPI levels in users of the levonorgestrel-IUD or copper-IUD.

Kemmeren *et al*. explained the differences in free PS induced by various oral contraceptives by the interaction between PS and C4BP. C4BP binds protein S in a high affinity complex (39). They observed that total PS was decreased by oral contraceptives containing desogestrel / ethinylestradiol but was hardly affected by oral contraceptives containing levonorgestrel / ethinylestradiol and that both oral contraceptives equally lowered C4BP. As a result, free PS levels increase in users of levonorgestrel / ethinylestradiol and decrease in users of desogestrel / ethinylestradiol.

Free PS forms a complex with free TFPI and acts as cofactor of TFPI through the extrinsic pathway (17;19-22). Inherited or acquired PS deficiency causes concomitant decreased free TFPI levels (20). A possible explanation could be that binding of free TFPI to free PS protects it from proteolytic degradation or slows down the clearance of free TFPI. So a decrease of free PS will then be accompanied by a decrease in free TFPI. The effect of hormonal contraceptives on free PS is probably only part of the mechanism of the increased risk of venous thrombosis since the effect on free TFPI-levels is usually larger than the effect on free PS. Due to this fact it is likely that TFPI production or release is also influenced by hormonal contraceptives; both proteins share the endothelium as common production site. In addition, the secretion of TFPI from endothelial cells might be coupled to PS secretion, as has recently been discovered for PS and the beta chain of C4BP by Carlsson *et al*. (20;45).

In conclusion, we observed that users of the levonorgestrel-IUD have similar levels of free PS and higher levels of free TFPI as non-users. Users of oral contraceptives containing drospirenone / ethinylestradiol, cyproterone actetate / ethinylestradiol and desogestrel / ethinylestradiol had lower free TFPI and free PS levels than users of oral contraceptives containing levonorgestrel / ethinylestradiol. A negative association between the thrombotic risks as reported in recent literature and free PS and free TFPI levels was observed. Our study confirms the hypothesis that the differences in APC resistance induced by hormonal contraceptives can be partly explained by different effects on free TFPI and free PS levels. Future studies are indicated to unravel the mechanism of the reduction of free PS and free TFPI during use of hormonal contraceptives and the exact mechanism of their relation with the risk of venous thrombosis.

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